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Ovarian endometrioma disrupts cumulus cell vitamin D receptor mRNA expression

Ramazan Ozyurt[1], Aret Kamar[2]
[1] Department of Obstetrics and Gynecology, Bakırköy Dr. Sadi Konuk Research and Training Hospital
[2] Department of Obstetrics and Gynecology, Private Istanbul IVF-Unit, Istanbul, Turkey

Abstract

Aim: To determine the vitamin D receptor (VDR) mRNA expression pattern in cumulus cells (CCs) isolated from metaphase II (MII) oocytes retrieved from patients with unilateral endometrioma during controlled ovarian stimulation (COS).

Material and Methods: Twelve unilateral unoperated endometrioma patients selected for in vitro fertilization-intracytoplasmic sperm injection (IVF-ICSI) were included in the study. For the control group, the left ovaries of 12 women undergoing IVF/ICCI treatment due to male factor infertility were chosen. Flexible GnRH antagonist protocol for controlled ovarian stimulation was applied to both groups. A total of 99 CC samples, 28 in the endometrioma group, 34 in the contralateral ovary group, and 37 in the control group, were subjected to qRT-PCR analysis was conducted to assess mRNA expression of VDR transcript 2 (VDR-X2).

Results: CC-VDR-X2 expression of the disease-free contralateral ovary appeared to be equivalent to the control group. mRNA expression levels of CC-VDR-X2 in patients diagnosed with endometrioma were significantly lower than the mRNA expression of the disease-free contralateral ovary and control group. A significant 2.31-fold downregulation was observed in the CC-VDR-X2 mRNA expression of the endometrioma group when compared to the CC-VDR mRNA expression of the contralateral ovary. The expression of CC-VDR-X2 mRNA in MII oocytes of the endometrioma-affected ovary was down-regulated 2.63-fold compared to the CC-VDR-X2 mRNA expression of the control group. 2PN zygote rates of endometrioma patients with low VDR-X2 levels were found to be similar to MII oocytes with normal VDR-X2 expression in the contralateral and control groups.

Discussion: Unilateral endometrioma may cause subfertility by blocking CC-VDR mRNA expression.

Keywords

Endometrioma, Cumulus Cell, Vitamin D Receptor, RT-PCR

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Corresponding Author: Ramazan Ozyurt, Department of Obstetrics and Gynecology, Bakırköy Dr. Sadi Konuk Research and Training Hospital, Istanbul, Turkey.
E-mail: atasagun02@hotmail.com P: +90 532 748 34 90
Corresponding Author ORCID ID: https://orcid.org/0000-0001-6822-2222
Other Authors ORCID ID: Aret Kamar, https://orcid.org/0000-0002-4737-6895
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Introduction

Endometrioma is a disease characterized by the presence of one or multiple cysts in one ovary or both, which histologically and functionally mimic eutopic endometrium, appearing in approximately one-third of women with endometriosis [1]. Infertility due to endometriomas has been attributed mainly to the adverse effects of cysts on oocytes' developmental capacity and, less commonly, to endometrial dysfunction [2-5] Mechanical stress caused by endometriomas, depending on their number, size, and location, can harm the both quantity and quality of oocytes by disrupting the blood supply and innervation of developing follicles [1, 3]. If endometrioma disrupts the normal anatomy of the fallopian tubes, it may negatively affect sperm-oocyte interaction and embryo transport [6]. In addition to the mechanical effect, free iron within the cyst may cause inflammation and embryotoxicity, leading to a decrease in the count number and quality of oocytes along with impaired embryo development [7, 8]. Despite all these data, the available data are not clear on how endometriomas affect oocyte developmental capacity. Early follicular atresia due to local inflammation and mechanical compression has been suggested as a possible mechanism [9]. However, the fact that cysts are removed via surgery did not significantly change the reproductive outcome bringing us to the question that endometrioma might be a developmental defect of the oocyte pool [10].

Ovarian steroidogenesis and follicular development are regulated by numerous systemic hormones and local paracrine factors, which act through the two-cell, two-gonadotropin pathway [11]. Vitamin D is a secosteroid that plays a role in the balance of intra-follicular inflammation required for ovarian steroid synthesis and cumulus expansion via its nuclear receptor. There is a broad expression of the Vitamin D Receptor (VDR) not only in calcium metabolism tissues but also in cumulus cells (CCs) [11-13]. The VD-VDR complex, with its anti-inflammatory and immunomodulatory properties, might help regulate the intra-follicle inflammatory balance and steroidogenesis necessary for the development of a competent oocyte [13]. The lower risk of developing endometriosis in women with high serum 25(OH)VitD [14, 15] and lower VD levels in severe endometriosis compared to women with mild endometriosis [16] are clues to the relationship between VD and endometriosis. If the insufficiency of oocyte developmental capacity in endometrioma is due to steroid synthesis defect, VDR expression, which is key to ovarian steroidogenesis, must likewise be negatively affected. The way to test this hypothesis is to analyze VDR-mRNA expression in CC cells obtained from diseased and healthy ovaries in unilateral endometrioma. This study was planned to determine the VDR mRNA expression pattern in CCs isolated from metaphase II (MII) oocytes of patients with unilateral endometrioma during controlled ovarian stimulation (COS).

Material and Methods

A group of twelve patients who applied to the Istanbul IVF unit for in vitro fertilization-intracytoplasmic sperm injection (IVF-ICSI) with the diagnosis of unilateral unoperated ovarian endometrioma were included in the study. Participants were selected among patients applying to the Istanbul IVF unit.

After obtaining consent to collect cumulus samples from the same center, patient-informed consent was also obtained. A comparison was made between the affected and the intact contralateral gonads in terms of cumulus cell VDR mRNA expression. The control group consisted of the left ovaries of 12 women who were set to undergo IVF/ICSI for male factor infertility. The twelve participants in the control group were identified from infertile patients who were age-matched with the endometrioma group and had both ovaries intact (endometrioma-free). The criteria for inclusion specified women under 35, undergoing IVF-ICSI, having no infertility cause besides endometrioma, possessing one or more ultrasound-diagnosed unilateral endometriomas, confirmed over two cycles, and no prior endometrioma surgeries. The criteria for exclusion were: a history of surgical intervention for either unilateral or bilateral endometrioma, surgery for a non-endometriotic benign ovarian cyst, diagnosis of bilateral endometrioma, and patients over 35 years of age.

Endometrioma diagnosis was established using transvaginal ultrasonography conducted between days 2 and 3 of the cycle, as previously stated [17] the numbers of antral follicles in the diseased and intact ovaries were also recorded. Endometrioma diameter was calculated by taking the average of the lengths of three perpendicular diameters4,5. Flexible GnRH antagonist protocol for controlled ovarian stimulation was applied to both groups. rFSH (Gonal-f, Merck Group, Darmstadt, Germany) was started on days 2 and 3 of the spontaneous menstrual cycle. The initial dose of rFSH was determined according to the patient's age, AFC, and AMH values. Pituitary suppression was achieved using the GnRH antagonist Cetrotide (Merck Group, Darmstadt, Germany) which was started when the dominant follicle was ≥ 14 mm. Ovulation was triggered with recombinant hCG (Ovitrelle, Merck Group, Darmstadt, Germany) when two or more follicles were ≥ 18 mm. Oocytes were collected 36 hours after the ovulation trigger. COCs, gathered under ultrasound guidance, were then subjected to cumulus cell processing.

CCs were separated from COCs by both enzymatic and mechanical methods. COCs were subjected to COC classification after being placed in new Petri dishes: COC grade 1 refers to mature oocytes that possess the first polar body (MII stage), COC grade 2 indicates the absence of the first polar body (MI stage), and COC grade 3 indicates GV stage oocytes. Mature oocytes (MII) were the sole source of CCs used for qRT-PCR. After two hours of incubation, each COC was denudated first enzymatically with Hyase and then mechanically with pipettes. The collected CCs were transferred to RNA-later-containing tubes and stored until total RNA extraction. CC samples of the ovary containing endometrioma were labeled CCMII-end (+) and CC samples of the contralateral ovary were labeled CCMII-end (-). The vials containing the left ovarian CC samples of the women in the control group were labeled as CCMII-con (Table 1). A total of 99 CC samples, 28 in the endometrioma group, 34 in the contralateral ovary group, and 37 in the control group, were subjected to qRT-PCR.

Quantitative RT-PCR analysis of VDR-X2

The mRNA expression of the VDR transcript 2 (VDR-X2) gene was analyzed using qRT-PCR. Total RNA isolation was performed with PureLink Total RNA Mini Kit (Invitrogen). Total RNA

concentration was measured with a Qubit Fluorometer. cDNAs were amplified by qRT-PCR with sequence-specific primers. Glyceraldehyde-3-phosphate dehydrogenase was preferred as a reference gene. Primers used for qRT-PCR had the following sequences: VDR-X2: Forward 5'-ACATTGCTTTGCTTGCCTCC-3', Reverse 5'-ACGTTCCGGTCAAAGTCTCC-3',
GAPDH: Forward 5'-GAAGATGGTGATGGGATTTC-3',
Reverse 5'-GAAGGTGAAGGTCGGAGTC-3'.

Statistical Analysis

The analysis of the data was carried out with IBM SPSS Statistics Version 22.0 for Windows (IBM Corp., Armonk, NY, USA). Independent samples t-test was employed for variables that displayed a normal distribution, whereas the Kruskall-Wallis test was used for those with non-normal distribution. Mean ± standard deviation is used to present continuous variables. $p<0.05$ was considered significant. Relative gene expression was measured following the 2-ΔΔCt formula. The presence of a statistical difference in VDR-X2 mRNA expression was evaluated using one-way ANOVA, followed by Tukey's multiple comparison test. CC-VDR-X2 mRNA expressions of endometrioma and contralateral disease-free ovaries were normalized by taking the CC-VDR-X2 mRNA expression of the control group as 1.

Ethical Approval

This study was approved by the Ethics Committee of Bakirkoy Dr. Sadi Konuk Education and Research Hospital Clinical Research Ethics Committee (Date: 2024-06-24, No: 2024-04-35/161).

Results

Table 2 displays the demographic characteristics of the participants. It was highlighted that the mean age (28.4±3.21 years vs. 27.5±3.81, p=0.233) infertility duration, AFC, and serum levels of AMH were similar in both groups. A significant reduction in serum VD levels was observed in the endometrioma group when compared to the control group. There was no significant difference between groups in terms of total gonadotropin dose used and total number of oocytes collected. The average uni-lateral endometrioma diameter was measured as 31.97±1.29 mm. CC-VDR-X2 expression levels of the disease-free contralateral ovary were found to show similarity to the control group (1.01 ± 0.22 vs. 0.88± 0.23, p = 0.556). In patients with endometrioma showed CC-VDR-X2 mRNA expression levels that were significantly lower than the mRNA expression of the disease-free contralateral ovary (0.38± 0.06 vs. 0.88 ± 0.23, p<0.002) and control group (0.38± 0.06 vs. 1.01 ± 0.22, p<0.001). A significant 2.31-fold downregulation was observed in the CC-VDR-X2 mRNA expression of the endometrioma group when compared to the CC-VDR mRNA expression of the contralateral ovary (Figure 1). CC-VDR-X2 mRNA expression of the MII oocytes of endometrioma ovary was down-regulated 2.63-fold compared to the CC-VDR-X2 mRNA expression of the control group. The COC morphologies of the endometrioma group with low VDR mRNA expression and the control and contralateral ovary groups with normal VDR expression were similar. Low VDR-X2 expression did not cause CC loss, darkening, or compaction. Fertilization rates after ICSI were similar in endometrioma and control groups. 2PN zygote rates of endometrioma patients with low VDR-X2 levels were similar to normal VDR-X2-expressing MII oocytes of the contralateral and control groups. In the endometrioma

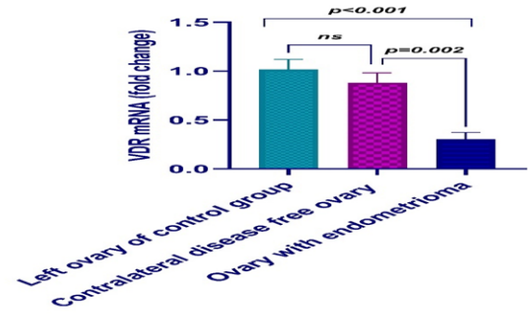

Figure 1. Graphical representation of CC-VDR-X2 expression levels of endometrioma, contralateral ovary, and control group. CC-VDR-X2 mRNA expression of the endometrioma group was approximately 2.5-fold lower than the expression levels of both control groups (Data are expressed as mean ± SEM. ns., not significant)

Table 1. Total number of CC samples submitted to qRT-PCR

Participants	COS protocol	Retrieved total oocytes and oocyte maturation stages	Number of CCMII samples
Ovary with endometrioma (n=12)	GnRH antagonist with r-hCG trigger	37 (28 MII, 6 MI, 3 GV)	28
Contralateral disease free ovary (n=12)		40 (34 MII, 4 MI, 2 GV)	34
Left ovary of control group (n=12)	GnRH antagonist with r-hCG trigger	44 (37 MII, 4 MI, 3 GV)	37
Total number of CC samples submitted to qRT-PCR			99

Table 2. Comparison of demographic characteristics of unilateral endometrioma and control groups

	AMH (ng/mL)	AFC	Size of endometrioma (mm)	Retrieved total oocyte	2PN zygotes (%)	Vitamin D (ng/mL)
1. Ovary with endometrioma (n=12)	2.14±1.20	2.98±1.43	31.97±1.29	3.18±2.33	71.4	10.34±3.09
2. Contralateral disease free ovary (n=12)		3.24±2.09		3.44±3.20	76.4	
3. Control group (left ovary, n=12)	2.48±1.09	3.69±2.41		3.87±2.10	78.3	14.71±4.26

group, the 2 PN zygote rate was 71.4%, while it was 76.4% in the contralateral ovary group, and 78.3% in the control group.

Discussion

Studies on how endometriomas affect oocyte quality and development have been mostly done using granulosa cells [18, 19]. It is thought that endometriomas exert their adverse effects on oocyte developmental capacity through more than one mechanism. One possible cause is that the malfunctioning of the two-cell, two-gonadotropin pathway due to the inflammatory follicular microenvironment negatively affects ovarian steroidogenesis [20, 21]. Cumulus granulosa cells effectively use vitamin D and nuclear vitamin D receptors during estrogen and progesterone synthesis [12]. Our analysis in this study is the first to examine the CC-VDR mRNA expression patterns of patients who underwent IVF/ICSI due to unilateral endometrioma. CC-VDR levels of endometrioma ovaries showed a 2.3-fold downregulation compared to the contralateral ovary. Similarly, CC-VDR levels of the endometrioma group showed a 2.6-fold downregulation compared to healthy controls without endometrioma. Our findings are of clinical importance as they provide the first evidence that CC-VDR expression, which is involved in ovarian steroid synthesis, is defective in the presence of endometrioma. Consistent with our findings, endometriomas are known to disrupt estradiol synthesis by reducing P450 aromatase expression in GCs [3]. For the follicle to reach metaphase II and gain competence, estradiol, a steroid, is necessary. [19]. The reduced estradiol levels in the follicular fluid, as compared to the control group are important evidence that endometrioma causes ovarian steroidogenesis defects [22]. Many studies have suggested that endometriomas disrupt ovarian steroidogenesis through mechanical action and the toxic effect of the ferrous content of the cyst [1-3]. In addition to the mechanical and ferroptotic effects of cysts, the decrease in CC-VDR expression in the presence of endometrioma may also contribute to the steroid synthesis defect.

How endometriomas lead to VDR expression defects is still unclear. In a physiological follicular cycle, CC-VDR expression increases in the presence of active VD. VD-VDR complex activation increases progesterone and estradiol synthesis by stimulating the production of 3β-hydroxysteroid dehydrogenase and insulin-like growth factor binding protein-1 [23]. The reduced serum VD levels in the unilateral endometrioma group, relative to the control group, might cause defects in CC-VDR expression. To clarify the influence of VD deficiency on CC-VDR, cumulus cell VDR expressions of patients with and without VD replacement before endometrioma cystectomy should be analyzed.

Since we found the AFC and total oocyte numbers collected in the endometrioma group to be similar to the contralateral ovary, we thought that unilateral endometrioma did not negatively affect the ovarian reserve. The effects of endometriomas on ovarian reserve may vary depending on whether the cyst is unilateral or bilateral. Although bilateral endometrioma causes a decrease in the total number of oocytes and poor embryo quality, the implantation, clinical pregnancy, or live birth rates are similar to healthy controls. In good agreement with this, a recent meta-analysis reported that endometriomas reduce the total number of oocytes retrieved, the yield of MII oocytes, and the embryo count [24]. Despite this, comparisons between unilateral endometrioma and contralateral normal ovaries in various studies revealed no differences regarding the total oocytes collected, MII oocytes, and embryo numbers [25].

We did not detect a significant difference between the COC morphology of the endometrioma group showing VDR expression defect and the COC morphology of the contralateral ovary and control groups. This finding suggests that VDR expression defect does not cause significant changes in cumulus expansion and corona radiata polarization. If VDR was directly involved in cumulus expansion, the COC morphology of endometrioma patients would be defective. In light of this information, we should not expect to obtain a different image from healthy controls when evaluating the COCs of endometrioma patients exhibiting VDR expression defects. However, normal COC morphology does not exclude the fact that steroid synthesis may be defective in patients with endometriomas with low VDR expression. The fact that fertilization rates after ICSI were similar in the endometrioma and control groups suggests that VDR expression defect is a pathology that can be treated with ICSI. Low VDR expression may contribute to endometrioma-associated infertility by disrupting the intra-follicle anti-inflammatory and immunomodulatory balance.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or compareable ethical standards.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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